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Additional Information

1 **Antilisterial and physical properties of biopolymer films containing**
2 **lactic acid bacteria.**
3
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5
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10
11 **Abstract**
12 Novel biopolymer films were developed and used to control *Listeria innocua* in an
13 artificially contaminated synthetized medium. Two hydrocolloids, sodium caseinate
14 (NaCas) and methylcellulose (MC), and two bacteriocin-producing lactic acid bacteria
15 (LAB), *Lactobacillus acidophilus* and *Lactobacillus reuteri*, were tested. Bioactive
16 cultures were added directly to the film forming solution and films were obtained by
17 casting. In order to study the impact of the incorporation of bacterial cells into the
18 biopolymer matrix, the water vapour permeability, optical and mechanical properties of
19 the dry films were evaluated. Furthermore, the survival of LAB and the antimicrobial
20 potential of bioactive films against *Listeria innocua* were studied. Results showed that
21 the use of lactic acid bacteria altered the film's physical properties. Films enriched with
22 bacterial cells exhibit higher gloss and transparency whereas no significant
23 modifications were observed in terms of tensile properties. These films were less-
24 effective water vapor barriers, since a significant increase can be observed in the WVP
25 values. As far as food safety is concerned, these films are an interesting, novel

26 approach. In refrigeration conditions, these films permit a complete inhibition of
27 *L.innocua* for a week. Viability of LAB was higher in sodium caseinate films, although
28 bacteriocin production was greater in polysaccharide matrix. The best results were
29 obtained for films made of methylcellulose, without differences between the two lactic
30 acid bacteria tested.
31 *Keyword: biopreservation, sodium caseinate, methylcellulose, Lactobacillus acidophilus, Lactobacillus reuteri,*
32 *mechanical properties, water vapour permeability.*
33

34 1. Introduction

35 There is increased consumer demand for a reduction in food additives and, particularly,
36 chemical preservatives, and, as a result, there is currently a great deal of research being
37 carried out into biopreservation and the use of natural antimicrobials for food
38 applications. Among these compounds, lactic acid bacteria (LAB) enjoy an advantage
39 as they are considered as GRAS (Generally Recognized As Safe). LAB can inhibit the
40 growth of different microorganisms, including bacteria, yeasts and fungi, through the
41 production of organic acids, hydrogen peroxide, enzymes, defective phages, lytic agents
42 and antimicrobial peptides, or bacteriocins (Alzamora et al., 2000).
43 LAB therefore offer great potential in food preservation. Among pathogens of interest
44 in food safety, the presence of the opportunistic psychrotroph foodborne pathogen
45 *Listeria monocytogenes* remains one of the major problems. This strain, which is able to
46 survive and grow at refrigeration temperature, is the causative agent of Listeriosis and is
47 lethal in 30 % of compromised individuals (Griffiths et al., 1989; Tauxe, 2002).
48 Previous studies have already proved the antilisterial efficacy of LAB in model systems
49 (Gialamas et al., 2010), in dairy products (Foulquie-Moreno et al., 2003; Liu et al.,
50 2008), in sea-food products (Concha-Meyer et al., 2011), as well as in meat products
51 (Maragkoudakis et al., 2009).

52 To guarantee food safety, the incorporation of lactic acid bacteria into biopolymer films
53 appears an interesting, novel approach. Cellulose derivatives are remarkable film
54 forming compounds. Not only are they biodegradable, odourless and tasteless (Krochta
55 and Mulder-Johnston, 1997) but they also exhibit good barrier properties against lipids,
56 oxygen and carbon dioxide (Nispero-Carriedo, 1994). Proteins also exhibit interesting
57 properties and, like polysaccharides, can replace conventional synthetic plastics.
58 Protein films generally have good mechanical properties and good barrier properties
59 against aroma and gases such as O_2 or CO_2 (Park and Chinnan, 1995; Miller and
60 Krochta, 1997; Gennadios, 2002; Letcher, 2007). Nevertheless, the incorporation of
61 lactic acid bacteria into biopolymer films can modify their functional properties such as
62 barrier or mechanical, which are crucial to assure the food protection against moisture
63 changes or mechanical damages. The food appearance can also be affected if optical
64 properties of the films result altered by the inclusion of bacteria.
65 The aim of this work was to evaluate how the functionality of sodium caseinate and
66 methylcellulose films was affected by the incorporation of lactic acid bacteria, through
67 the analysis of different physical properties (water vapor barrier, mechanical and optical
68 properties) as well as their antilisterial effect. Two bacteriocin producer strains are
69 compared, *Lactobacillus acidophilus* and *Lactobacillus reuteri*. *Listeria innocua*, a
70 non-pathogenic specie, was used instead of *Listeria monocytogenes,* since it has been
71 proven that this strain is physiologically similar to *L.monocytogenes* (Begot et al.,
72 1997).
73

74 **2. Materials and methods**

75 *2.1. Preparation of the bioactive films*

76 The film forming aqueous dispersions (FFD) contained 4 % (w/w) of methylcellulose
77 (MC, CAS 9004-67-5, Sigma-Aldrich, Madrid, Spain) or sodium caseinate (NaCas,
78 CAS 9005-46-3, Sigma-Aldrich, Madrid, Spain) and glycerol (Panreac Quimica, S.A.,
79 Castellar Del Vallés, Barcelona, Spain) as plasticizer, using a hydrocolloid:glycerol
80 mass ratio of 1:0.25. Polymers were dissolved in distilled water under continuous
81 stirring at 25 °C. After dispersion, glycerol was added and FFD were homogenized in a
82 rotor-stator ultraturrax DI25 at 13.500 rpm for 4 min. FFD were degasified at 7 mbar at
83 room temperature under vacuum (Wertheim, Germany).
84 Two lactic acid bacteria, *Lactobacillus acidophilus* (Casenfilus®, CASEN Fleet, Spain)
85 and *Lactobacillus reuteri* (CasenBiotic®, CASEN Fleet, Spain), were added to NaCas
86 and MC films.
87 The selection of the strains was based on their antimicrobial activity against
88 *L.monocytogenes* observed in preliminary experiments (data not shown). Microbial
89 cultures were regenerated by transferring 1 g of commercial preparation into 10 mL of
90 MRS broth and incubated at 37 ºC overnight. A 10 µl aliquot from overnight culture
91 was again transferred into 10 mL of MRS broth and grown at 37 ºC for 24 h. Cells were
92 harvested by centrifugation at 6000 rpm for 20 min and washed twice with sterile
93 tryptone phosphate water (Scharlab, Barcelona, Spain). Lactic acid bacteria were
94 incorporated by adding the bacterial cells preparation into the FFD (0.1 mL per 37.5 g).
95 FFD were then placed under magnetic stirring for 5 min.
96 A casting method was used to obtain the bioactive films. FFD were poured onto a
97 framed and levelled polytetrafluorethylene (PTFE) plate (ϕ = 15 cm) and were dried in
98 atmospheric conditions (25 ºC, 60 % relative humidity) for approximately 48 hours.
99 Film thickness was controlled by pouring the amount of FFD that will provide a surface
100 density of solids in the dry films of 56 g/m^2 in all formulations. The average thickness

101 of the obtained films was 78 ± 6 μm. Dry films were peeled off the casting surface and
102 preconditioned for one week in desiccators at 5 ºC and 75 % relative humidity (RH)
103 prior to testing. These values of temperature and RH were chosen to simulate similar
104 storage conditions to those given when coated foodstuffs with these film formulations
105 were stored under refrigeration. The moisture content of the films was determined after
106 equilibration. To this end, film samples were dried in triplicate at 60 °C for 24 h in a
107 natural convection oven and for 24 h more in a vacuum oven. Moisture content was
108 determined from de sample weight loss.
109
110 *2.2. Water vapour permeability*
111 Water vapour permeability (WVP) was measured in dry film discs (ϕ = 7 cm),
112 previously equilibrated at 75 % RH and 5 ºC, according to the "water method" of the
113 ASTM E-96-95 (ASTM, 1995), using Payne permeability cups (Elcometer SPRL,
114 Hermelle /s Argenteau, Belgium). Deionised water was used inside the testing cup to
115 achieve 100 % RH on one side of the film, while an oversaturated sodium chloride
116 solution was used to control the RH (75 %) on the other side of the film. During WVP
117 testing, the side of the film in contact with the PTFE plate during the drying step of film
118 preparation was placed in contact with that part of the test cup having the highest RH. A
119 fan placed on the top of the cup was used to reduce resistance to water vapour transport.
120 To calculate WVTR, the slopes of the steady state period of weight loss as a function of
121 time were determined by linear regression. For each type of film, WVP measurements
122 were replicated three times and WVP was calculated according Mc Hugh et al. (1993).
123
124 *2.3. Mechanical properties*

125 Mechanical properties were measured by using a Texture Analyser TA-XT-plus (Stable
126 Micro Systems, Surrey, UK), with a 50 N load cell equipped with tensile grips (A/TG
127 model). Sample films, previously equilibrated at 75 % RH and 5 ºC, were cut into 25.4
128 mm wide and 100 mm long strips, according to the ASTM D-882 standard (ASTM,
129 2001). Grip separation was set at 50 mm and cross-head speed was 50 mm/min. Tensile
130 strength (TS) and percentage of elongation (% E) at break, and elastic modulus (EM)
131 were evaluated in eight samples from each type of film.
132
133 *2.4. Optical properties*
134 Gloss was measured using a flat surface gloss meter (Multi-Gloss 268, Minolta,
135 Langenhagen, Germany) at an angle of 60 º with respect to the normal to the film
136 surface, according to the ASTM standard D523 (ASTM, 1999). Prior to gloss
137 measurements, films were conditioned in desiccators at 5 ºC and 75 % RH. Gloss
138 measurements were performed over a black matte standard plate and were taken in
139 quintuplicate. Results were expressed as gloss units, relative to a highly polished
140 surface of standard black glass with a value close to 100.
141 The transparency of the films was determined through the surface reflectance spectra in
142 a spectrocolorimeter CM-3600d (Minolta Co, Tokyo, Japan) with a 10 mm illuminated
143 sample area. Measurements were taken from three samples in each formulation by using
144 both a white and a black background. The transparency was determined by applying the
145 Kubelka-Munk theory for multiple scattering to the reflection spectra. As each light flux
146 passes through the layer, it is affected by the absorption coefficient (K) and the
147 scattering coefficient (S). Film transparency was evaluated from the internal
148 transmittance (Ti), as indicated by Hutchings (1999), from the reflectance of the sample
149 on a white background of known reflectance and on an ideal black background, as

150 described by (Pastor et al., 2010). CIE Lab coordinates were determined through the
151 infinite reflectance spectra.
152

153 *2.5. Viability of lactic acid bacteria during the storage of the films*

154 The viability of lactic acid bacteria was studied in NaCas and MC films just prepared
155 and periodically for 30 days. The films were stored in Petri Dishes inside of desiccators
156 at 5 ºC and 75 % relative humidity and every 7 days they were removed from the Petri
157 dishes and placed in a sterile plastic bag with 100 ml of tryptone phosphate water
158 (Scharlab, Barcelona, Spain). The bag was homogenized for 2 minutes in a Stomacher
159 blender (Bag Mixer 400, Interscience). Serial dilutions were made and then poured onto
160 MRS agar. Plates were incubated for 48 hours at 37 ºC before colonies were counted.
161 All tests were run in duplicate.
162

163 *2.6. Antimicrobial activity of the films against Listeria innocua*

164 *2.6.1. Bacterial strain*

165 Stock culture of *Listeria innocua* (CECT 910), supplied by Colección Española de
166 Cultivos Tipos (CECT, Burjassot, Spain), was kept frozen (-25 ºC) in Tryptone Soy
167 Broth (TSB, Scharlab, Barcelona, Spain) supplemented with 30 % glycerol (Panreac,
168 Barcelona, Spain). Culture was then regenerated by transferring a loopful into 10 mL of
169 TSB and incubated at 37 ºC overnight. A 10 µl aliquot from overnight culture was again
170 transferred into 10 mL of TSB and grown at 37 ºC to the end of the exponential phase of
171 growth. Subsequently, this appropriately diluted culture was used for the inoculation of
172 the agar plates in order to obtain a target inoculum of 10^2 UFC/cm^2.
173

174 *2.6.2. Antimicrobial effectiveness of films*

175 The methodology followed for the determination of antimicrobial effectiveness of films
176 was adapted from Kristo et al. (2008). Aliquots of Tryptone Soy Agar (TSA, Scharlab,
177 Barcelona, Spain) (20 g) were poured into Petri dishes. After the culture medium
178 solidified, properly diluted overnight culture from *L.innocua* was inoculated on the
179 surface and the different films (containing or not *L.plantarum*) of the same diameter as
180 the Petri dishes were placed onto the inoculated surfaces. Plates were then covered with
181 parafilm to avoid dehydration and stored at 5ºC for 12 days. *L.innocua* and lactic acid
182 bacteria counts on TSA plates were examined immediately after the inoculation and
183 periodically during the storage period.
184 The agar was removed aseptically from Petri dishes and placed in a sterile plastic bag
185 with 100 ml of tryptone phosphate water (Scharlab, Barcelona, Spain). The bag was
186 homogenized for 2 minutes in a Stomacher blender (Bag Mixer 400, Interscience).
187 Serial dilutions were made and then poured onto MRS agar and PALCAM agar. Plates
188 were incubated for 48 hours at 37 ºC before colonies were counted. All tests were run in
189 duplicate.
190
191 *2.7. Bacteriocin detection*
192 A bicinchoninic acid protein assay kit was used (Sigma Aldrich, Spain). Pure
193 biopolymer films and films enriched with LAB were placed in a sterile plastic bag with
194 10 mL of physiological water and homogenized for 2 minutes in a Stomacher blender.
195 The homogenates were centrifuged and the supernatants were used to determine
196 bacteriocin concentration.
197
198 *2.8. Statistical analysis*

199 Results were analysed by multifactor analysis of variance with 95 % significance level
200 using Statgraphics®Plus 5.1. Multiple comparisons were performed through 95 % Least
201 Significant Difference intervals (LSD).
202

203 **3. Results and discussion**

204 *3.1. Water vapour permeability*

205 Water vapour permeability (WVP) of the films is one of the most important parameters
206 defining film functionality. This property quantifies the film effectiveness in preventing
207 the moisture exchanges between the food and the environment, which affect the product
208 firmness and appearance. So, low WVP values are desirable to minimize weight losses
209 of coated products which directly affect product quality. The WVP and moisture content
210 of the films are shown in Table 1. The RH gradient was chosen to simulate the
211 environmental conditions of coatings applied to fresh products, such as meat, cheese or
212 fish, cold stored. The WVP values of pure hydrocolloid films with plasticizer were
213 16.7±0.7 and 14.29±0.16 $g.mm.kPa^{-1}.h^{-1}.m^{-2}$ for sodium caseinate and methylcellulose
214 films, respectively.

215 These values are in the order of those previously reported for these polymer films,
216 (Pinotti et al., 2007; Fabra et al., 2010) taking into account the influence of
217 experimental conditions (temperature, RH gradient) (Greener and Fennema, 1989) and
218 the different amount of plasticizer. A higher content of plasticizer enhances material
219 flexibility, WVP and equilibrium moisture content (Audic and Chaufer, 2005;
220 Hernandez-Izquierdo and Krochta, 2008). The amount of glycerol used (ratio 1:0.25 of
221 polymer:glycerol) was necessary to overcome the brittleness of protein films. Only a
222 slight difference between the two biopolymer matrices in WVP values was observed.

223 As can be seen in Table 1, the addition of lactic acid bacteria significantly decreased
224 film barrier properties regardless of the type of the hydrocolloid. This could be
225 attributed to the introduction of discontinuities in the film matrix due to the presence of
226 bacteria. These discontinuities make the film matrix more open to mass transfer.
227 Nevertheless, since the mass of microbial cells is relatively small, the increase in WVP
228 is moderate. For NaCas films, WVP values increased regardless of the nature of the
229 strain, although for MC films the WVP increase occurs more notably when
230 *Lactobacillus reuteri* was incorporated.
231 Equilibrium moisture content of NaCas and MC films were similar and, in both cases,
232 an increase in this value was observed when lactic acid bacteria were incorporated in the
233 matrix. This increase could be attributed to the greater water retention capacity of the
234 microorganisms to ensure their survival. The greater film moisture content will also
235 contribute to the greater values of WVP of the films.
236
237 *3.2. Mechanical behaviour*
238 Mechanical properties were measured in terms of the percentage of elongation (E%) and
239 tensile strength (TS) at break and elastic modulus (EM). TS represents the film's
240 resistance to elongation or its stretching capacity and EM is a measure of the stiffness of
241 the film. The values are shown in Table 1. Pure methylcellulose films were
242 mechanically more resistant to fracture and more stretchable (greater TS, EM and E%
243 values) than pure sodium caseinate films. Results were in line with those published by
244 Turhan and Sahbaz (2004) and Fabra et al. (2009) for MC and sodium caseinate films,
245 respectively.
246 The mechanical response of both kinds of films presented similar trends when LABs
247 were incorporated into the matrix: a reduction of elastic modulus and tensile strength at

248 break without significant changes in the film stretchability. The elastic modulus and
249 tensile stress reduction were more marked in methylcellulose films and in sodium
250 caseinate films when *Lactobacillus reuteri* was incorporated. In this sense, Gialamas et
251 al. (2010) reported the same trends in sodium caseinate films. The relative low
252 proportion of added cells explains their small repercussion on film mechanical
253 properties.
254 The effect of the incorporation of microbial cells on the mechanical behavior of the
255 films is coherent with the introduction of discontinuities (microbial cells) in the polymer
256 matrix, which implies a reduction in the cohesive forces in the polymer network with
257 the subsequent losses of mechanical resistance. Nevertheless, the effects are not relevant
258 due to the fact that a relatively low number of discontinuities (cells) were introduced in
259 each case.
260
261 *3.3. Optical properties*
262 The optical properties of the films, color coordinates, transparency and gloss, were
263 evaluated, since these properties have a direct impact on the appearance of the coated
264 product. As can be seen in Figure 1, color coordinates were affected ($p > 0.05$) by the
265 presence of the bioactive cultures. No significant differences were observed between
266 sodium caseinate and methylcellulose films without microbial cells. The addition of
267 lactic acid bacteria provoked an increase in the film lightness (L^*) in both kinds of
268 films. In sodium caseinate films, the addition of microorganisms provoked an increase
269 in color saturation (C_{ab}^*) whereas the opposite occurred in methylcellulose films. The
270 same opposite behavior was observed for the film hue: this became more yellow in
271 NaCas films and greener in MC films when microbial cells were incorporated.

272 Nevertheless, the differences induced by microbial cells were very small from the
273 practical point of view.
274

275 Film transparency was evaluated through the internal transmittance, Ti (0-1, theoretical
276 range). An increase in Ti can be assumed as an increase in transparency (Hutchings,
277 1999). The spectral distribution of Ti (400–700 nm) is shown in Figure 1d.
278 Methylcellulose films were significantly more transparent than NaCas films (higher
279 values of Ti). In all cases, the addition of microbial cells implied an increase in the film
280 transparency regardless of the strain. This increase was similar (approximately 7 %) for
281 both kinds of films and could be related with the grater water content of the films.
282 Gloss values of the films measured at incidence angle values of 60 º are reported in
283 Table 1. No differences were observed between the two hydrocolloid matrices. The
284 addition of protective culture to the MC and NaCas matrix led to a slight increase of the
285 gloss. The gloss of the films is related with the surface morphology reached during film
286 drying. In general, the smoother the surface, the higher the gloss (Ward and
287 Nussinovich, 1996). In this sense, the increase of the film gloss when microbial cells
288 were incorporated could be due to the cell separation near the film surface, which
289 modifies the refraction index in this zone affecting surface optical properties. Therefore,
290 the addition of bioactive culture significantly improves the appearance of NaCas and
291 MC films. Indeed films become more transparent with higher lightness and gloss.
292

293 *3.4. Viability of lactic acid bacteria and bacteriocin concentration during storage of the*
294 *films*

295 The viability of *Lactobacillus acidophilus* and *Lactobacillus reuteri* added to MC and
296 NaCas films was tested trouhhout a storage period of one month at 5 °C and 75 % RH.

297 Microbial counts as a function of the storage time are shown in Figure 2. As can be
298 seen, the viability of *L. acidophilus* was greater than that of *L.reuteri* in both polymer
299 matrices. For *L.reuteri*, a significant reduction of the initial population was observed
300 during the first week of storage, which indicates that this strain is more sensitive to the
301 stress suffered during storage. By comparing the two hydrocolloid matrices, sodium
302 caseinate appears to be a more favourable environment for the survival of LAB.
303 Previous studies reported a similar positive effect of sodium caseinate films on the
304 survival of other lactic acid bacteria, *Lactobacillus plantarum* and *Lactobacillus sakei*
305 (Sánchez-González et al., 2013; Gialamas et al., 2010). It seems that during the drying
306 step of film preparation, the nature of the strain is the determining factor with respect to
307 bacterial survival. Regardless of the nature of the matrix, worse results were obtained
308 for *L.reuteri* in comparison with *L.acidophilus*. Counts for *L.reuteri* were lower than 3
309 log UFC / cm^2 in all films after 5 days storage, which indicates the great sensitivity of
310 this strain to the lack of nutrients and to the decrease of the water content. The kind of
311 polymer of the film also plays an important role in microbial survival. As described
312 previously by Sánchez-González et al. (2013) a greater microbial viability was obtained
313 in protein films than in films from cellulose derivatives.
314

315 The amount of bacteriocin produced by *Lactobacillus acidophilus* and *Lactobacillus*
316 *reuteri* in the different films was evaluated in newly-prepared films and troughout the
317 storage period (one month at 5 °C and 75 % RH). The values of bacteriocin
318 concentration are shown in Table 2. Bacteriocin production clearly differs depending on
319 the nature of the hydrocolloid: protein or polysaccharide. The concentration of
320 bacteriocin was higher for films based on methylcellulose, regardless of the strain
321 added. These observed differences remain during the storage period. The amount of

322 bacteriocin increases throughout storage time for both NaCas and MC films containing
323 *L.acidophilus* and *L.reuteri*. The greater bacteriocin production was obtained in
324 methylcellulose films, without significant differences between the two LABs.
325 Therefore, the nature of the film hydrocolloid was a determining factor for both the
326 viability of the protective culture and bacteriocin production.
327
328 *3.5. Antilisterial activity*
329 The antimicrobial activity of the developed films against *L.innocua* was tested in a
330 synthetic non-selective medium (TSA) stored at 5 °C. Counts of *L.innocua* are shown
331 in Figure 3b.
332 Pure NaCas and MC films, with no lactic acid bacteria, were used as control samples.
333 As is shown in Figure 3b, *Listeria innocua* population increased from 3.18 to 6.18 log
334 CFU/cm^2 at the end of the storage period. As expected, pure protein and polysaccharide
335 films were not effective at reducing the *L.innocua* growth, since no significant
336 differences were observed in microbial growth with respect to TSA plates. All films
337 containing bioactive cultures exhibited a significant antilisterial activity since, during
338 the first week of storage, a reduction of the initial microbial population was observed in
339 all cases. The two strains showed, therefore, bactericidal activity. After 3 storage days
340 the best results were obtained with methylcellulose films, but no differences among the
341 different bioactive films were observed after longer storage times. At the end of the
342 storage period (12 storage days), all films with *L.acidophilus* or *L.reuteri* led to a
343 reduction of the microbial growth of approximately 1.5 logs with respect to the control.
344
345 As is shown in Figure 3a, LAB added in NaCas films grew immediately after the film
346 came into contact with the surface of the medium, reaching a level in the order of 10^7

347 CFU/cm^2 after 3 storage days. Similar results were published by Gialamas et al. (2010)
348 for NaCas films with *Lactobacillus sakei*. However, for films based on polysaccharide,
349 different results were obtained. In this case, the initial population remained constant
350 during the first week and, after that, a slight decrease was observed. In this sense,
351 significant viability problems were observed for *L. reuteri* added in MC based films.
352 To understand antimicrobial effectiveness of polysaccharide and protein films
353 containing LAB, several factors must be considered. Even though all the developed
354 films presented an interesting antilisterial activity, MC based films were more effective
355 during the first three storage days. However, the viability of the strain decreased in this
356 matrix in line with an increase in the bacteriocin production, probably due to the fact
357 that the polysaccharide environment causes a greater stress in the metabolism of the
358 microbial cells (Nes et al., 1996; Gálvez et al., 2007). The greater concentration of
359 bacteriocins in the films contributes to enhance the antibacterial activity of the MC
360 films and so, it can be concluded that the antilisterial potential of the LABs present in
361 the films is essentially due to the action of the bacteriocins produced and not to the
362 possible growth competition between the strains.
363
364 **4. Conclusion**
365 The incorporation of microbial cells of LAB did not notably alter the films' tensile
366 properties whereas it improved the films' gloss and transparency. However, it provoked
367 a significant increase of the WVP values, regardless of the polymer and the strain used.
368 Sodium caseinate and methylcellulose films with glycerol showed themselves to be
369 effective carriers of *L.acidophilus* and *L.reuteri* bacterial cells, used as antimicrobial
370 agents. Indeed, the films with bioactive cultures presented interesting antilisterial
371 activity. The best results were obtained for methylcellulose films, regardless of the

372 lactic acid bacteria tested, which was related with a greater production of bacteriocins
373 caused by the fact that the polysaccharide medium was less suitable for the cell survival.
374

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380

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463 **Figure Captions**

464

465 **Figure 1.** Effect of the incorporation of bacterial cells on optical properties of
466 biopolymers films. Colour coordinates (a,b,c) and spectral distributions of the internal
467 transmittance (d). Mean values and 95 % LSD intervals.

468 **Figure 2.** Survival of lactic acid bacteria in biopolymer films throughout storage time at
469 5 °C and 75 % RH (□ MC + *Lactobacillus acidophilus*, ○ NaCas + *Lactobacillus*
470 *acidophilus*, ■ MC + *Lactobacillus reuteri*, ●NaCas + *Lactobacillus reuteri*). Mean
471 values and 95 % LSD intervals.

472 **Figure 3.** Survival of lactic acid bacteria in the films in contact with the culture media
473 (a) and effect of bioactive films on the growth of *Listeria innocua* (b) on TSA medium
474 stored at 5 ºC (□ MC + *Lactobacillus acidophilus*, ○ NaCas + *Lactobacillus*
475 *acidophilus*, ■ MC + *Lactobacillus reuteri*, ● NaCas + *Lactobacillus reuteri*, ◊ MC, Δ
476 NaCas and X control). Mean values and 95 % LSD intervals.

Figure 1

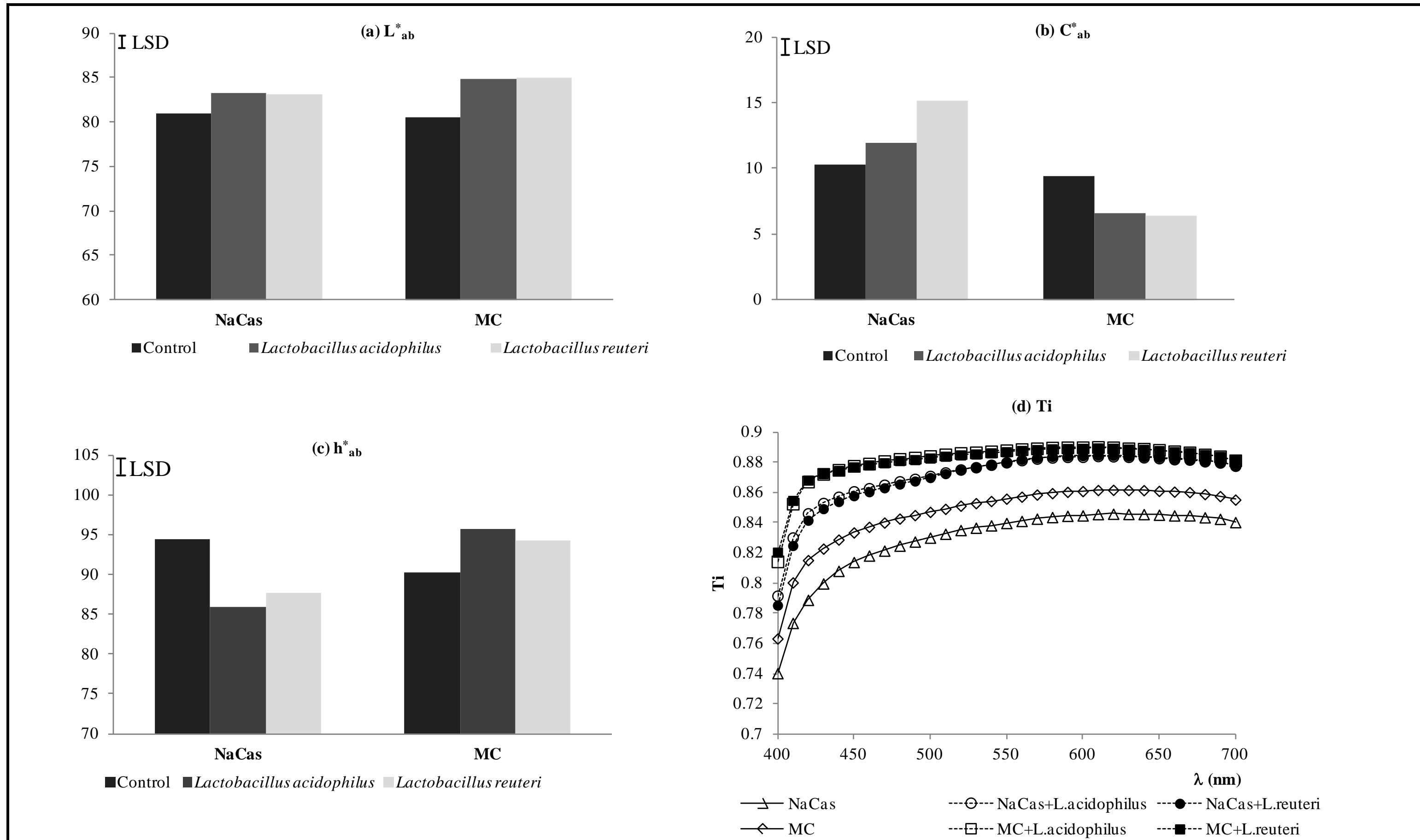

Figure 2

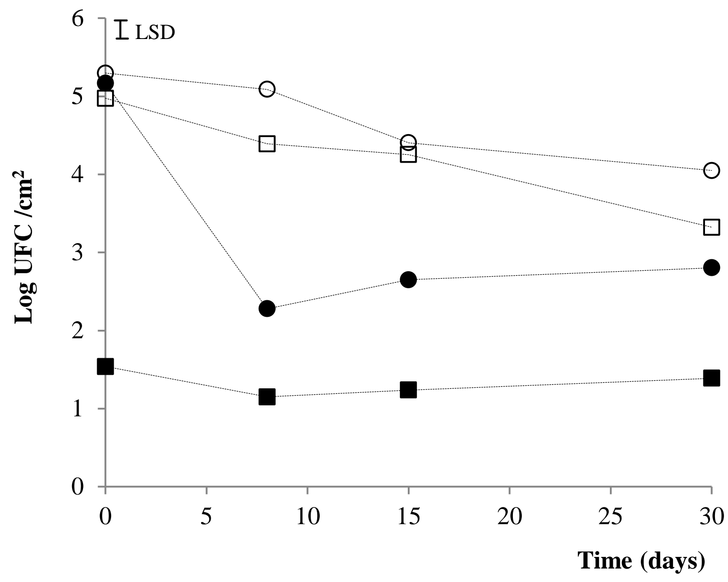

Figure 3

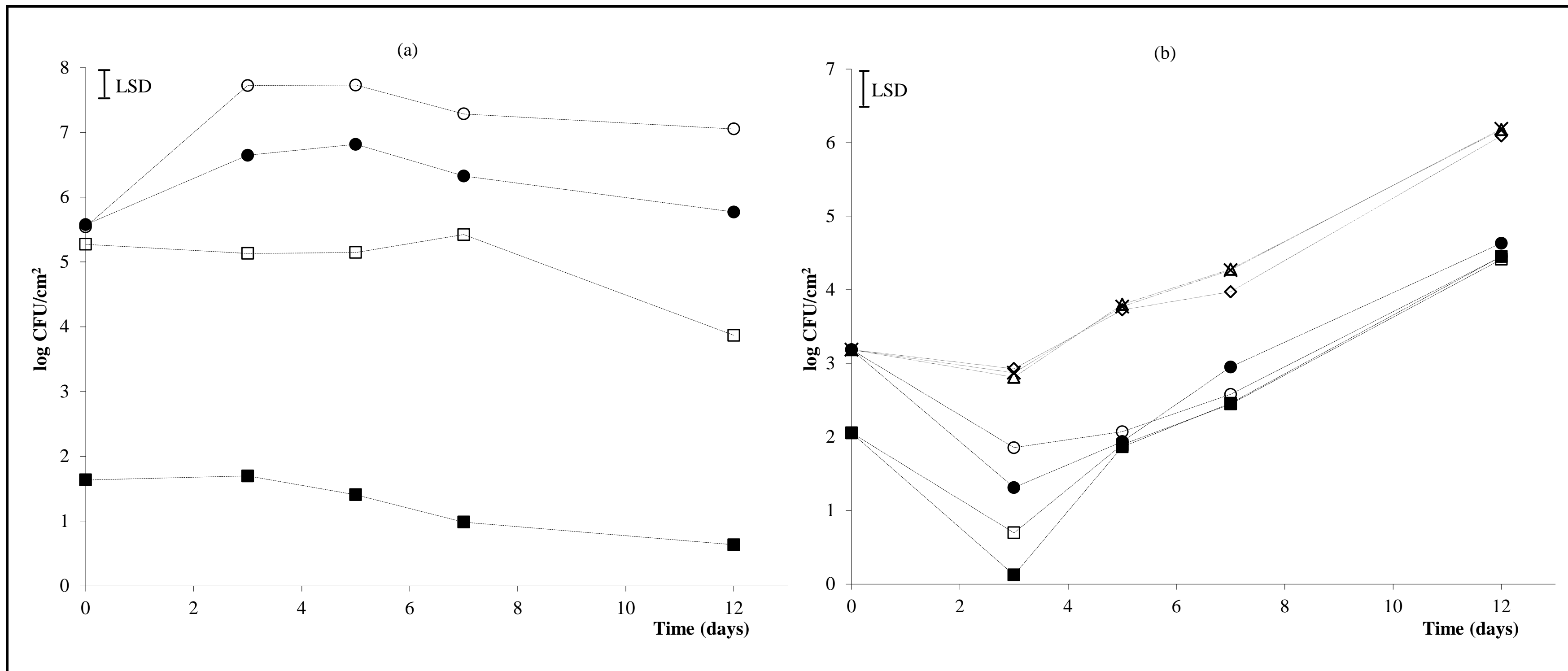

Table 1. Effect of the incorporation of lactic acid bacteria (*Lactobacillus acidophilus* and *Lactobacillus reuteri*) on moisture content, water vapour permeability, gloss and mechanical properties of biopolymer films.

Film	E (%)	TS (MPa)	EM (MPa)	Gloss 60 °	WVP ($g.mm.kPa^{-1}.h^{-1}.m^{-2}$)	Moisture content (g water. g $film^{-1}$)
NaCas	6.0 (0.3)[a]	11.2 (1.2)[a]	345 (79)[a]	30 (3)[ab]	16.7 (0.7)[a]	0.110 (0.003)[ab]
NaCas+*L.acidophilus*	5.8 (0.2)[a]	10.0 (0.8)[a]	318 (34)[a]	35 (2)[c]	20.9 (0.6)[c]	0.127 (0.006)[cd]
NaCas+*L.reuteri*	6.3 (0.2)[a]	6.9 (0.5)[b]	252 (23)[b]	33.4 (1.9)[bc]	19 (2)[c]	0.122 (0.008)[bc]
MC	29 (7)[b]	31.6 (0.8)[c]	532 (25)[c]	27.3 (1.3)[a]	14.29 (0.16)[b]	0.104 (0.008)[a]
MC+*L.acidophilus*	32.9 (1.3)[c]	30.7 (0.8)[c]	332 (35)[a]	33 (3)[c]	15.1 (0.5)[ab]	0.140 (0.007)[de]
MC+*L.reuteri*	33 (2)[bc]	24 (2)[d]	328 (21)[a]	32 (3)[bc]	19.8 (0.8)[c]	0.141 (0.005)[e]

[a, b,c,d,e] Different letters in the same column indicate significant differences among formulations ($p < 0.05$).

Table 2. Bacteriocin concentration in films containing lactic acid bacteria at different storage times: 0, 8, 15 and 30 days, at 5 °C and 75 % RH.

Films	**Bacteriocin (mg/mL)**			
	t_0	t_8	t_{15}	t_{30}
NaCas+*L.acidophilus*	238 (5)^{aw}	236.5 (1.6)^{aw}	265.1 (1.6)^{ax}	277.7 (1.4)^{ay}
NaCas+*L.reuteri*	272.9 (1.1)^{bw}	268.5 (1.6)^{bx}	283.9 (1.2)^{by}	281.0 (0.7)^{bz}
MC+*L.acidophilus*	301.1 (0.2)^{cw}	292.2 (1.9)^{cx}	311.3 (0.5)^{cy}	315.6 (0.4)^{cz}
MC+*L.reuteri*	294 (2)^{dw}	292.4 (0.9)^{cx}	313.3 (0.7)^{dy}	315.2 (1.2)^{cy}

$^{a,\,b,c,d}$ Different letters in the same column indicate significant differences among formulations ($p < 0.05$).
^{w,x,y,z} Different letters in the same file indicate significant differences among time for a same formulation ($p < 0.05$).